Published in final edited form as:
Mutat Res. 2009 October 2; 669(1-2): 27–35. doi:10.1016/j.mrfmmm.2009.04.012.

Defective interaction between Pol2p and Dpb2p, subunits of DNA polymerase epsilon, contributes to a mutator phenotype in *Saccharomyces cerevisiae*

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Summary

Most of the prokaryotic and eukaryotic replicative polymerases are multi-subunit complexes. There are several examples indicating that noncatalytic subunits of DNA polymerases may function as fidelity factors during replication process. In this work, we have further investigated the role of Dpb2p, a noncatalytic subunit of DNA polymerase epsilon holoenzyme from *Saccharomyces cerevisiae* in controlling the level of spontaneous mutagenesis. The data presented indicate that impaired interaction between catalytic Pol2p subunit and Dpb2p is responsible for the observed mutator phenotype in *S. cerevisiae* strains carrying different mutated alleles of the *DPB2* gene. We observed a significant correlation between the decreased level of interaction between different mutated forms of Dpb2p towards a wild-type form of Pol2p and the strength of mutator phenotype that they confer. We propose that structural integrity of the Pol epsilon holoenzyme is essential for genetic stability in *S. cerevisiae* cells.

Keywords

DNA polymerase; Pol epsilon holoenzyme (Pol ε HE); Spontaneous mutagenesis; Fidelity of DNA replication; Protein-protein interaction; *DPB2* (*YPR175W*) and *POL2* (*YNL262W*) genes of the yeast *Saccharomyces cerevisiae*

1. Introduction

Errors occurring during DNA replication constitute one major source of spontaneous mutagenesis [1]. In most organisms, the fidelity of DNA replication is determined by three highly conserved processes: base selection by DNA polymerase, proofreading of the misinserted nucleotides by 3′→5′ exonuclease, and post-replicative DNA mismatch repair

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Conflict of Interest Statement
The authors declare that there are no conflicts of interest.

system (MMR) [2]. For years studies of the mechanisms controlling DNA replication errors have been mainly concentrated on MMR and on the role of subunits housing DNA polymerase and 3′→5′ exonuclease activities. However, most of the prokaryotic and eukaryotic replicative polymerases are multi-subunit complexes consisting of several different subunits. For example, *Escherichia coli* DNA polymerase III holoenzyme (Pol III HE), the enzyme responsible for chromosomal DNA replication, is 17-subunit protein complex [3,4]. In the budding yeast *Saccharomyces cerevisiae*, the nuclear chromosome replication is performed by three DNA polymerase holoenzymes: polymerase alpha (Pol α HE), polymerase epsilon (Pol ε HE), and polymerase delta (Pol δ HE) consisting of at least four (Pol α HE: Pol1p, Pol12p, Pri1p and Pri2p; Pol εHE: Pol2p, Dpb2p, Dpb3p and Dpb4p) and three (Pol δ HE: Pol3p, Pol31p and Pol32p) subunits [5]. With regard to the fidelity of the replication process, the Pol2p subunit of Pol ε and Pol3p of Pol δ are of particular importance. Both are catalytic subunits that possess DNA polymerase and 3′→5′ exonuclease activity. Strains carrying exonuclease deficient Pol ε (*pol2-4*) or Pol δ (*pol3-01*) are mutators [6]. However, the fidelity of DNA replication may depend not only on the intrinsic accuracy of the polymerase and the efficiency of the proofreading performed by the 3′→5′ exonuclease, but also on the appropriate structural and functional interactions between the catalytic and noncatalytic subunits within DNA polymerase holoenzymes.

There are several examples suggesting that noncatalytic DNA polymerase subunits may influence the DNA replication fidelity, but the contribution of accessory subunits remains poorly characterized. In *Escherichia coli*, mutations in the *dnaX* gene, which encodes the τ subunit of Pol III HE, lead to a mutator phenotype [7,8]. In *S. cerevisiae*, mutations in *PRI1* and *PRI2*, the primase subunits of Pol α: primase complex, enhance the spontaneous mutation rate [9]. The absence of Pol32p, a nonessential subunit of Pol δ HE, lead to the increase frequency of deletions of sequences flanked by short direct repeats. The *pol32*Δ mutants also exhibit synergistic increase in frameshift and base substitution mutations, when combined with the *msh2*Δ allele [10]. Genetic data strongly suggest that at least two, Dpb2p and Dpb3p, accessory subunits of *S. cerevisiae* Pol ε HE are required to maintain high fidelity of DNA replication. A *dpb3* Δ mutant displays a mutator phenotype [11]. Recently, we have characterized three temperature-sensitive *dpb2* alleles, which cause very strong mutator effect, even greater than that observed previously for Pol ε mutants defective in the 3′→5′ exonuclease proofreading activity (*pol2-4*) [12]. These limited data indicate that noncatalytic subunits of DNA polymerases may function as fidelity factors during replication process.

In the work reported here, we have further investigated the role of Dpb2p, an essential protein for yeast cells, in controlling the level of spontaneous mutagenesis. The present study has been undertaken to confirm that proper interaction between Dpb2p, a noncatalytic subunit, and the catalytic Pol2 subunit of Pol ε HE is important in maintaining the overall genetic stability in *S. cerevisiae* cells. Data presented indicate that impaired communication between the catalytic Pol2 subunit and the Dpb2 protein is responsible for the mutator phenotype in *S. cerevisiae* strains carrying mutated alleles of the *DPB2* gene. Interestingly, we observed a significant correlation between the decreased ability of different mutated forms of Dpb2p to interact with Pol2p and the strength of mutator phenotype that they confer. We propose that structural integrity of Pol ε HE is essential for accurate chromosomal DNA replication in *S. cerevisiae* cells.

2. Materials and Methods

2.1. Media, strains and general methods

Yeast strains were grown in standard media [13]. Cells were grown nonselectively in YPD medium (1 % yeast extract, 1 % peptone and 2 % glucose). For yeast transformations and mutagenesis assays, yeast strains were grown in SD minimal medium (0.67 % yeast nitrogen

base without amino acids, 3 % glucose) supplemented with appropriate L-amino acids and nucleotides. To identify forward mutations in the *CAN1* locus, SD medium was additionally supplemented with L-canavanine [60 mg/L], an analog of arginine. SD medium supplemented with uracil and 0.1 % of 5-fluoroorotic acid (5-FOA) was used for selection of *ura3* mutants [14].

Strains with the wild-type *DPB2* and *dpb2* temperature-sensitive alleles are derivatives of the SC11 strain (*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 dpb2::kanMX4* [pMJDPB2]), described previously [12]. However, these strains bear the pGJ2 (*DPB2 HIS3*) or pMJ (*dpb2 HIS3*) centromeric plasmids instead of pMJDPB2 containing *DPB2* and *URA3* genes. For the two-hybrid assay, the Y190 strain (*MAT***a** *trp1-901 his3-200 leu2-3,112 ura3-52 ade2-101 lys2-801 gal4Δ gal80Δ cyh2 LYS2::GAL1*$_{\text{UAS}}$*-HIS3*$_{\text{TATA box}}$*-HIS3 URA3::GAL1*$_{\text{UAS}}$*-GAL1*$_{\text{TATA box}}$*-lacZ*) was used [15].

S. cerevisiae cells were transformed using lithium acetate/single-stranded carrier DNA/PEG method [16]. Isolation of plasmid DNA from yeast cells was performed using the method described by Hoffman and Winston [17].

2.2. Random mutagenesis of DPB2 and isolation of the temperature-sensitive dpb2 alleles

Random mutagenesis of the *DPB2* gene and selection of the temperature-sensitive *dpb2* alleles were previously described in details [12]. Briefly, a library of mutated variants of *DPB2* was created on a centromeric plasmid pRS313 (*HIS3*) [18] using the procedures of random mutagenesis. The SC11 strain (*dpb2::kanMX4* [pMJDPB2]) was transformed with the library of the mutated *DPB2* variants cloned into the pRS313 plasmid and incubated at 23°C for 7–10 days. To remove the pMJDPB2 (*DPB2 URA3*) plasmid, the transformants were transferred twice, using toothpicks, at 23°C onto plates additionally containing uracil and 5-FOA [14]. After the screening of about 15,000 Ura$^-$ colonies, several temperature-sensitive clones, unable to grow at 37°C, were identified. The DNA sequence of each mutant gene was determined by standard methods.

The pMJ111 and pMJ112 plasmids (Fig. 1) were constructed by replacing the 657-bp StuI-MunI and 390-bp ClaI-BamHI fragments of the wild-type *DPB2* gene on the pGJ2 plasmid [11], with the corresponding mutated fragments excised from pMJ106. Similarly, the pMJ113 plasmid containing *dpb2-113* allele was constructed by replacing 657-bp StuI-MunI fragment of pGJ2 with the one from pMJ107 [12]. The temperature sensitivity of the newly constructed *dpb2* mutants was determined as described above.

2.3. Construction of two-hybrid plasmids

Plasmids for the two-hybrid system are based on pKF75 and pKF80 multi-copy vectors [12], which are bearing a sequence encoding Gal4p DNA-binding (BD$_{GAL4}$) or transcription activation domain (AD$_{GAL4}$), respectively. These plasmids contain a backbone of pGBT9 or pGAD424 (Clontech), respectively, and a new polylinker compatible with a common series of bacterial and shuttle (*E. coli*/yeast) cloning vectors, *i.e.* pBluescript (Stratagene) and pRS [18]. The pKF133 (pKF75-*dpb2::plomba*), pKF134 (pKF75-*DPB2*), and pKF164 (pKF80-*pol2*$_{(K2090\text{-}I2222)}$) plasmids were described previously [12]. To create fusions of mutated *dpb2* alleles with the BD$_{GAL4}$ sequence, the pKF133 vector was used, whose short (73 bp) *plomba* sequence was subsequently replaced with subcloned fragments of the respective alleles. The plasmids bearing truncated alleles of *DPB2* are schematically shown in Fig. 5 (in the Results section) and have been constructed as follows: The pKF135 vector (pKF75-*dpb2*Δ$_{(H318\text{-}P581)}$) has been created by subcloning of the 740-bp PstI-EcoNI fragment of pKF134 into PstI/EcoNI-linearized pKF133, while pKF136 (pKF75-*dpb2*Δ$_{(W70\text{-}I313)}$) is a result of ligation of the 801-bp EcoNI-ClaI fragment of pKF134 into EcoNI/ClaI-linearized

pKF133. The pKF168 (pKF75-*dpb2*$_{(E43\text{-}F557)}$) and pJK4 (pKF75-*dpb2*$_{(M1\text{-}D583)}$) plasmids have been constructed respectively by subcloning of the 1539-bp EcoRI-EcoRI and 1765-bp BamHI-ClaI parts of pKF134 into pKF75 at the same sites. In the case of pKF168, the clone bearing *DPB2* fragment in the same orientation as the BD$_{GAL4}$ sequence was chosen. Both hybrid genes of pKF168 and pJK4 use the in-frame STOP codon from the 3′-terminal region of the polylinker. Finally, the 420-bp EcoRI-SalI and 341-bp ClaI-SalI fragments of pKF134 were separately cloned into pKF75 yielding pJK2 (pKF75-*dpb2*$_{(E556\text{-}I689)}$) and pJK3 (pKF75-*dpb2*$_{(I582\text{-}I689)}$) plasmids, respectively.

2.4. Measurement of spontaneous mutation frequency, calculation of mutation rates and statistical analysis

The frequency of spontaneous mutagenesis was measured at the *CAN1* locus. Any mutation that inactivates the arginine permease, encoded by *CAN1*, results in the resistance to L-canavanine (Can^R), an analog of arginine. To measure the spontaneous Can^R frequencies, 10 independent cultures were grown in liquid SD medium (3 ml) supplemented with required amino acids and nucleotides. The cultures were grown to stationary phase at 23°C. Yeast cells were collected by centrifugation, washed with water, and resuspended in water. Aliquots of the appropriately diluted strains were plated on non-selective plates, whereas undiluted cultures were plated on selective plates supplemented with L-canavanine [60 mg/L]. Plates were incubated for 7–10 days at 23°C and colonies were counted. Each experiment was repeated three times. Mutant frequency was determined by dividing the median mutant count by the median of the total cell count. The mutation rates were calculated as described previously [12,19].

The *p*-values for significance of differences between the strain carrying wild-type *DPB2* and respective strains bearing mutated variants of *DPB2* were determined using the nonparametric Mann-Whitney criterion and the STATMOST software (DataMost, Salt Lake City, UT). Such statistical calculations were used for data obtained from survival, mutation rate, and two-hybrid experiments.

2.5. Two-hybrid assay

To monitor protein-protein interactions, the yeast two-hybrid system was used [20]. The yeast strains were grown for 1 day at 30°C in SD medium supplemented with required amino acids and nucleotides. The cultures were diluted 10 times with fresh SD medium and incubated for 36 h at 23°C or 24 h at 30°C or at 33°C. The *lacZ* genetic reporter was then used to indicate the interactions, which were determined using a quantitative *in vitro* β-galactosidase assay with *O*-nitrophenyl galactoside (ONPG) as a substrate [21].

2.6. Immunoblot analysis of yeast extracts

Yeast strains were grown at 23°C in SD medium supplemented with required amino acids and nucleotides until OD$_{600nm}$ reached 0.75 units. Cells from 10-ml cultures were collected by centrifugation, and pellets were frozen in liquid nitrogen and stored at −80°C. The cells were resuspended in a buffer (100μl) containing 40 mM HEPES pH 8.0, 150 mM NaCl, 1 mM DTT, 2 mM PMSF and the mix of the protease inhibitors (SIGMA), and disrupted by vortexing 6 times for 30 sec. with 50μl of the 0.4–0.6 mm glass beads (Sartorius). The cell debris was pelleted by centrifugation twice for 15 min. at 4°C. The samples were prepared by heating protein extracts at 100°C for 5 min. in the 1x sample-loading buffer (50 mM Tris-Cl pH 6.8, 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol and 100 mM β-mercaptoethanol). Proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences). To detect both the wild-type Dpb2p (78 kDa) expressed from a chromosome of the Y190 strain and the BD$_{Gal4}$-Dpb2 fusion proteins (c.a. 97 kDa) expressed from the two-hybrid plasmids, rabbit anti-Dpb2p antibodies were used (kindly provided by

Hiroyuki Araki, National Institute of Genetics, Shizuoka, Japan). Next, the blots were incubated with the ImmunoPure Goat Anti-Rabbit IgG antibodies conjugated with the horseradish peroxidase (HRP; PIERCE). Bands were visualized using chemiluminescent substrates for HRP (SuperSignal WestPico, PIERCE) and Fluorchem SP Imager (Alpha Innotech). The same blots were also probed with anti-Hts1p (histydyl-tRNA synthethase, 60 kDa) antibodies as a gel loading control. The resulting bands were quantified using ImageQuant 5.2 (Molecular Dynamics).

3. Results

3.1. Cell viability and mutator phenotype of the dpb2 mutants

To further understand the *dpb2*-dependent mutator effect and the mechanism by which the Dpb2 protein may control the level of spontaneous mutagenesis, we have undertaken a genetic analysis of 10 new *dpb2* mutants based on their inability or partial ability to grow at 37°C. Seven of them (*dpb2-104* to *dpb2-110*) were obtained by mutagenic PCR of the *DPB2* gene (Materials and Methods and [12]). DNA sequence analysis of newly isolated mutant *dpb2* alleles showed that all contained multiple missense mutations within the *DPB2* gene (Fig. 1). Three other alleles (*dpb2-111*, *dpb2-112* and *dpb2-113*) were constructed by subcloning of appropriate fragments from *dpb2-106* and *dpb2-107* (Fig. 1 and Materials and Methods). During this study several new alleles of the *DPB2* gene were constructed by subcloning different fragments of the originally isolated temperature-sensitive ones (data not shown). However, only two of them, *dpb2-112* and *dpb2-113*, (Fig. 1B) retained the temperature-sensitive phenotype (Fig. 2) and therefore were chosen for further study. Temperature sensitivity of ten *dpb2*Δ strains, carrying mutated alleles of *DPB2* on a plasmid, is shown in Fig. 2. Eight strains were inviable at 37°C. The viability of the *dpb2-109* strain was 10- to 20-fold lower than the wild-type one (Fig. 2). Only the strain carrying the *dpb2-111* alelle, grew as well as the *DPB2* control strain.

We next measured cell viability using plating efficiency assay to determine if there was any growth defect at 23°C (Fig. 3). Interestingly, we observed that different mutants affect cell viability differently. Five mutants: *dpb2-106*, *dpb2-107*, *dpb2-110*, *dpb2-112* and *dpb2-113* show statistically significant loss of viability.

Next, to determine whether the presence of *dpb2* alleles leads to the mutator phenotype, we determined the frequency of spontaneous mutagenesis in all strains (Fig. 4A). We used a forward mutant rate assay that detects mutations inactivating arginine permease, encoded by the *CAN1* gene, as described in Materials and Methods. Mutations in *CAN1* that inactivate Can1p, result in resistance of the yeast cells to the presence of L-canavanine (the Can^R phenotype) in the medium. Nine of the *dpb2* alleles exhibited a statistically significant increase of mutation rate ranging from ~2-fold (*dpb2-108* and *dpb2-109*) to 8-fold (*dpb2-113*). Only *dpb2-111* showed no increase in mutability.

3.2. Interaction of mutant Dpb2 proteins with Pol2p

In a previous paper [12], we described three mutator *dpb2* alleles and proposed that the mutator phenotype could be attributed to the lack of proper interaction between Dpb2p and Pol2p. To test this hypothesis we used yeast two-hybrid system. We showed previously that the C-terminal fragment of Pol2p (a.a. 2090-2222) cloned into Gal4p activation domain-coding vector (pKF80) interacted strongly and specifically with entire Dpb2 protein cloned into Gal4p binding domain-coding vector (pKF75) [12]. We cloned all ten *dpb2* mutant DNA genes described above into pKF133 (see Materials and Methods), and interaction with Pol2p were assessed by ability to transactivate the *GAL1-lacZ* reporter in the *S. cerevisiae* Y190 strain expressing AD_{Gal4p}-$Pol2p_{(K2090-I2222)}$ at 23°C and 33°C (the highest permissive temperature

for the Y190 strain). The data in Table 1 and Fig. 4B indicate that nine mutant Dpb2 proteins show decreased interaction with Pol2p. Strain carrying the *dpb2-111* allele, which grows at 37°C in BY background as *DPB2* control strains, shows no increase of mutability and interacts at 23°C with Pol2p as strongly as *DPB2*. Interestingly, the remaining mutants exhibit 2- to 6-fold weaker interaction (*dpb2-104*, *dpb2-108* and *dpb2-109*), dramatically reduced interaction (*dpb2-107*, *dpb2-110* and *dpb2-112*) or even lack of interaction (*dpb2-105*, *dpb2-106* and *dpb2-113*) with Pol2p, even at 23°C. At 33°C (Table 1) the *dpb2-111* allele showed normal Dpb2p-Pol2p interaction, in contrast to the nine remaining *dpb2* mutants, which showed residual interaction (*dpb2-108* and *dpb2-109*) or no interaction.

To rule out the possibility that lack of interaction is attributed to the lack of expression of the mutant Dpb2 proteins, we have analyzed expression of six representative Dpb2p variants in the low (Dpb2p-104), medium (Dpb2p-105 and Dpb2p-110), and high (Dpb2p-106, Dpb2p-107 and Dpb2p-112) frequency mutator category by Western blotting (Fig. 4C). As an internal control for the chromosomal expression level of Dpb2p we used Y190 strain with the empty (without the *DPB2* gene) pKF75 plasmid. Using anti-Dpb2p antibodies we were able to detect both the wild-type Dpb2p (78 kDa) expressed from the XVI chromosome of the Y190 strain and the BD_{Gal4}-Dpb2 fusion proteins (c.a. 97 kDa) expressed from two-hybrid plasmids. All Dpb2p proteins tested are expressed at similar levels, excluding the possibility that impaired Dpb2p-Pol2p interaction were due to the reduced expression of particular Dpb2p variant. The Western blot analysis presented in Fig. 4C shows that the Y190 strain carrying plasmids encoding the BD_{Gal4}-Dpb2 mutant fusion proteins possess additional cross-reacting protein band migrating between wild-type Dpb2p and BD_{Gal4}-Dpb2 fusion proteins. This band is also present in a strain carrying the BD_{Gal4p} fusion with wild-type Dpb2p. Currently we may only speculate that this band represents either a specific degradation product of the BD_{Gal4}-Dpb2 fusion protein or a postranslationally modified wild-type form of Dpb2p, perhaps due to the increased intracellular concentration of the hybrid protein.

In summary, we observe a negative correlation between the strength of mutator phenotype (Fig. 4A) and relative strength of the Dpb2p-Pol2p interaction (Fig. 4B).

3.3. Deletion mapping of the Dpb2p region engaged in interaction with the C-terminal fragment of Pol2p

The Dpb2 protein is 689 amino acids long. To identify the region of Dpb2p that is responsible for interaction with the C-terminal fragment (a.a. 2090-2222) of Pol2p, a series of truncated forms of the Dpb2p were fused to the BD_{Gal4p} (Fig. 5A) and their ability to interact with $Pol2p_{(K2090\text{-}I2222)}$ was analyzed (Fig. 5B). As controls we used two previously described mutant Dpb2 proteins: Dpb2p-100 (L284P T345A) that caused strong mutator phenotype and did not interact detectably with $Pol2p_{(K2090\text{-}I2222)}$, and Dpb2p-102 (T345A), which did not cause changes in the level of spontaneous mutagenesis and interacted with $Pol2p_{(K2090\text{-}I2222)}$ as well as the wild-type Dpb2 protein [12]. By examining the activation of the reporter gene, we found that none of the truncated BD_{Gal4}-Dpb2 fusion proteins is able to interact with the AD_{Gal4}-$Pol2_{(K2090\text{-}I2222)}$ fusion protein (Fig. 5B). A plausible explanation for these results is that the integrity of the entire Dpb2p sequence is essential for the Pol2p-Dpb2p complex formation.

4. Discussion

Saccharomyces cerevisiae DNA polymerase ε holoenzyme is a heterotetrameric complex consisting of Pol2p (the catalytic subunit), Dpb2p, Dpb3 and Dpb4 [22,23,24,25]. We initiated studies to assess the possible involvement of the Dpb2 subunit in the fidelity of DNA replication by isolation of the temperature-sensitive *dpb2* mutants [12]. Our previous results have shown that three tested *DPB2* alleles: *dpb2-100*, *dpb2-101* and *dpb2-103*, which carry mutations in

different regions of the *DPB2* gene, exhibited strong mutator phenotype. We have also shown that a significant portion of the *dpb2*-dependent replication errors are proofread by the 3′→5′ exonuclease of Pol ε and those *dpb2*-dependent mutations, both base substitutions and frameshifts, are subject to correction by mismatch repair system [12]. Based on the results obtained we have concluded that Dpb2p is essential not only for cell viability, but also for fidelity of DNA replication. We have hypothesized that the observed decrease in fidelity of DNA replication, in *S. cerevisiae* strains carrying mutations within the *DPB2* gene, could be due to the aberrant subunit interactions within Pol ε HE. The results presented here are consistent with this hypothesis. We have shown that the strength of the mutator phenotype observed in 10 new *dpb2* mutants is inversely proportional to the strength of the interaction between Dpb2p and Pol2p. Although caution should be used in interpretation of the two-hybrid results, our work implies that conformational changes in the *dpb2* mutants lead to the observed differences in Dpb2p-Pol2p interaction and indicate a direct casual linkage to the mutator phenotype observed in strains carrying these mutated *dpb2* alleles. Our previous data supports this conclusion, but also indicates that there may be additional factors contributing to mutagenesis as one of the previously tested alleles, *dpb2-103*, causes a strong mutator phenotype, but Dpb2p103-Pol2p interaction is reduced only 2-fold at 23°C [12]. Our data indicate that structural integrity of Pol ε HE is an important contributor to the accurate chromosomal DNA replication in *S. cerevisiae* cells.

It is possible that some component of the mutator phenotype observed in the *dpb2* mutants is dependent on increased participation of low-fidelity translesion polymerases, such as Pol ζ, in processing mismatched primer terminus [26, Jaszczur *et al.* in preparation]. We also cannot rule out the possibility that the mutator effect of the *dpb2* mutants is partially due to the participation of DNA Pol ε in other DNA transactions.

At the beginning of our study, we tried to model the 3D structure of Dpb2p using computational techniques. We used profile Hidden Markov Models as implemented in HHpred [27] to detect homologies among experimentally resolved 3D structures collected in PDB database [28]. Although this is a highly sensitive method (compared to PSI-Blast or even profile-sequence comparisons), we were unable to determine any true homologs in PDB. Only a fragment of Dpb2p, between amino acids ~400–689 was found to have distant homology to a known 3D structure, which was unfortunately not enough to build a reliable model. Recently, a solution structure of the N-terminal 75-amino acid domain of human Pol ε subunit B (Pole2p) revealed homology to the C domain of AAA+ proteins, however, the remainder of the Pole2 protein appears to have an unknown fold [29].

Since we were unable to get any information concerning the possible 3D structure of Dpb2p from *in silico* modeling approach we have decided to isolate a library of temperature-sensitive *DPB2*-mutated strains by random mutagenesis procedure. All alleles contained multiple base substitutions in the *DPB2* gene resulting in amino acid changes (Fig. 1B). As amino acid substitutions occurred at random along the entire length of Dpb2p (Fig. 1A), we were unable to identify regions important for structure and function. Such a random pattern of mutations may suggest that entire fold of protein is responsible for its activity and that different amino acid changes perturb proper folding of Dpb2p. This interpretation is consistent with our failure to detect a discrete Dpb2p-Pol2p binding domain in Dpb2p (Fig. 5). Failure of Dpb2p fragments to fold into a conformation able to interact with Pol2p is not necessarily surprising. A good example of the requirement of the integrity of the entire sequence for specific protein-protein interaction comes from a study concerning interactions of *E. coli* polymerase V subunits, UmuC and UmuD' [30]. The UmuC protein is 422 amino acids long and is able to interact strongly and specifically with UmuD'. However, even removal of 13 amino acids from the N terminus of UmuC or 26 amino acids from C terminus of UmuC completely eliminates the ability of UmuC to interact with UmuD'. These results suggest that the integrity of the entire

amino acid sequence of Dpb2p and UmuC protein is critical for Dpb2p-Pol2p and UmuC-UmuD' complex formation, respectively.

The important role of proper protein-protein interactions between DNA polymerase holoenzyme subunits in controlling the activity and/or fidelity of DNA replication have been shown previously in yeast [9,31,32,33], human [34], and in prokaryotic cells [30,35]. Human DNA polymerase γ, responsible for mitochondrial DNA replication, is composed of a catalytic subunit p140 (*POLG* gene product) and an accessory subunit p55 (*POLG2* gene product) [36]. Mutations in *POLG* cause several mitochondrial diseases including progressive external ophthalmoplegia (OPE) [37]. However, Longley *et al.* [34] described a pathogenic mutation (G451E) in *POLG2* associated with autosomal dominant OPE. Biochemical characterization of the G451E p55 showed no physical or functional interaction of mutant p55 protein with the p140 catalytic subunit. The authors suggest that impaired assembly of the Pol γ HE, due to the G451E mutation, leads to stalling of the mtDNA replication fork and, in consequence, an increased frequency of mtDNA deletions. In *E. coli* the core of Pol III HE contains three subunits:α (*dnaE* gene product possessing DNA polymerase activity), ε; (*dnaQ* gene product possessing 3′→5′ exonucleolytic activity) and θ (structural subunit encoded by *holE*); which form a heterotrimeric complex α-ε-θ [4,5]. Several mutators, which carry mutations in the *dnaQ* gene, have been isolated. The DnaE-DnaQ interaction for three *dnaQ* mutator alleles (*MutD5*, *DnaQ926* and *DnaQ49*) has been characterized using the two-hybrid approach [35]. Two of the DnaQ mutant proteins (MutD5 and DnaQ926) are fully proficient in binding to DnaE subunit. This result is consistent with the localization of mutations responsible for mutator phenotype of the *mutD5* and *dnaQ926* strains in the catalytic Exo I motif and their dominant mutator phenotype [38]. In contrast, the recessive *dnaQ49* mutation results in 6-fold weaker interaction with DnaE. This result suggests that the mutator phenotype observed in the *dnaQ49* strain may be due to defective communication between the polymerase subunit (DnaE) and the proofreading subunit (DnaQ) of Pol III HE in *E. coli*.

Additionally to 23°C, in two-hybrid experiment we also tested the ability of different Dpb2 mutant proteins to interact with Pol2p at 33°C (the highest permissive temperature for the Y190 strain). At this temperature we observe the temperature-dependent loss of binding of the mutated Dpb2p variants to Pol2p (Table 1). We may speculate that this result is consistent with the observed temperature-sensitive phenotype of the cells carrying the mutated *dpb2* alleles (Fig. 2). However, Dpb2p subunit specified by *dpb2-105*, *dpb-106* and *dpb2-113* have barely detectable, if any, level of binding not only at 33°C but also at 23°C, where they are able to form colonies, though evincing a lower plating efficiency (Table 1 and Fig. 3). This result, together with spontaneous mutagenesis experiments, may indicate that the Dpb2p-Pol2p interaction is physiologically relevant to the observed mutator phenotype, but lack of the Dpb2p-Pol2p interaction is not necessarily responsible for the temperature sensitivity observed in different mutated *dpb2* strains. Currently we may only speculate that at 23°C there is residual Dpb2p-Pol2p interaction and that this interaction is sufficient to allow *dpb2* cells to grow at this temperature. Alternatively, we can not rule out the possibility that temperature sensitivity of *dpb2* strains is observed because Dpb2p is involved in the other essential DNA replication-associated reactions and these processes are affected in *dpb2* strains. This issue is being currently investigated in our laboratory.

The identification of an accessory subunit of DNA Pol ε in *S. cerevisiae*, as a genetic stability enhancer, may also have important implications for our understanding of the etiology of genetic instability in humans. The human DNA polymerase ε has similar subunit composition as yeast Pol ε. The human Pol ε HE is composed of a 261-kDa catalytic subunit (p261, a *POLE1* gene product) and three associated subunits p59 (*POLE2*), p17 (*POLE3*) and p12 (*POLE4*) [39,40, 41]. The human p59 subunit, ortholog of Dpb2p, has 26 % overall identity and 44 % similarity to the yeast Dpb2 protein [40]. We may expect that some mutations in the *POLE2* gene can

perturb p261-p59 interaction leading to a mutator phenotype even in the heterozygous state. The situation could be similar to that observed in *POLG2*$_{(G451E)}$-dependent autosomal dominant OPE. It has been suggested that haplotype insufficiency of the wild-type p55 protein causes OPE by reducing availability of functional Pol γ HE in mitochondria [34]. Also the haplotype insufficiency of DNA polymerase β is probably responsible for the observed increase cancer risk and alter mortality rate in β-pol$^{+/-}$mice model [42].

In summary, we have asked the question what the significance of the interaction between Pol2p and Dpb2p has for the genomic stability. We have postulated previously that decreased communication between the Dpb2p and Pol2p subunits may influence the activity of catalytic Pol2p subunit and/or may change the processivity of Pol ε HE and/or may increase the probability of error-prone polymerases to participate in replication process [12]. The mechanisms that we have proposed are not mutually exclusive. It is possible that they all reflect situations *in vivo*. However, more detailed studies are required to understand the significance of Pol2p-Dpb2p interactions at the molecular level. Thus, on a more general level, our results provide a novel insight into the possible role of accessory subunits of DNA polymerase holoenzymes in controlling genome stability.

Acknowledgments

We thank Dr Zygmunt Cie la of the Institute of Biochemistry and Biophysics PAS (Warsaw, Poland) for critical reading of the manuscript, Dr Pawel Siedlecki (Institute of Biochemistry and Biophysics, PAS, Warsaw, Poland) for his efforts in modeling the Dpb2p structure, and Dr Hiroyuki Araki (Division of Microbial Genetics, National Institute of Genetics, Research Organization of Information and Systems, Shizuoka, Japan) for providing anti-Dpb2p antibodies. We thank also Dr Thomas L. Mason (Department of Biochemistry, University of Massachusetts, Amherst, MA) for anti-Hts1p antibody.

This work was supported by grant 2P04A05126 from the Polish Ministry of Science and Higher Education to M.J., K.F., P.J. and I.J.F; and by U.S. Public Health Service grant TW006463 [Fogarty International Collaboration Award (FIRCA)] to I.J.F., P.J. and J.L.C. K.F. research was additionally supported by grant N302 051 32/3925 from the Polish Ministry of Science and Higher Education.

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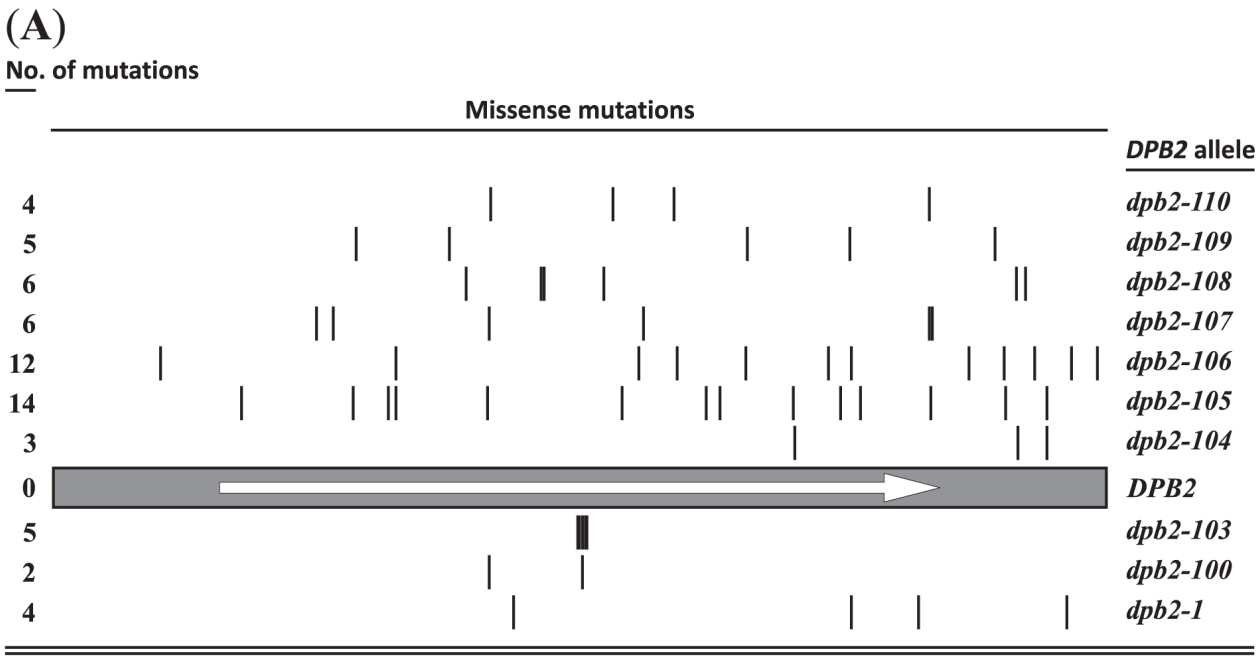


(B)

Plasmid	Amino acid substitutions	DPB2 allele
pMJ104	I484S L630S F649C	*dpb2-104*
pMJ105	S122G N195D F218L Y223C L283P S371P D426A Q435H F483L K514E S527P M573T I622T F649Y	*dpb2-105*
pMJ106	V69E Y223H D382G F407L I452M D506G K521E E598D P621S L641H I665V T682A	*dpb2-106*
pMJ107	K171E S182P L284P I385T M572V S574T	*dpb2-107*
pMJ108	T269A H318R Y320S I359V T629A I635V	*dpb2-108*
pMJ109	K197M N258S S453C C520L F615S	*dpb2-109*
pMJ110	L285W L365S N405I M572K	*dpb2-110*
pMJ111	Y223H (derivative of pMJ106)	*dpb2-111*
pMJ112	E598D P621S L641H I665V T682A (derivative of pMJ106)	*dpb2-112*
pMJ113	K171E S182P L284P (derivative of pMJ107)	*dpb2-113*

Figure 1. DNA sequence analysis of the *dpb2* mutated alleles

A. Distribution of missense mutations within the *dpb2* alleles.The *DPB2* open reading frame is schematically shown as a filled box; an arrow indicates the 5′ → 3′ direction of the coding DNA strand. The *dpb2* alleles used in this study and ones described previously [10,11] are shown over and below the box, respectively. Patterns of mutations of the particular *dpb2* alleles are presented as sets of vertical strokes (|). Each stroke marks an individual point mutation causing a.a. substitution.

B. Amino acid substitutions in the Dpb2p mutated variants obtained in this study.

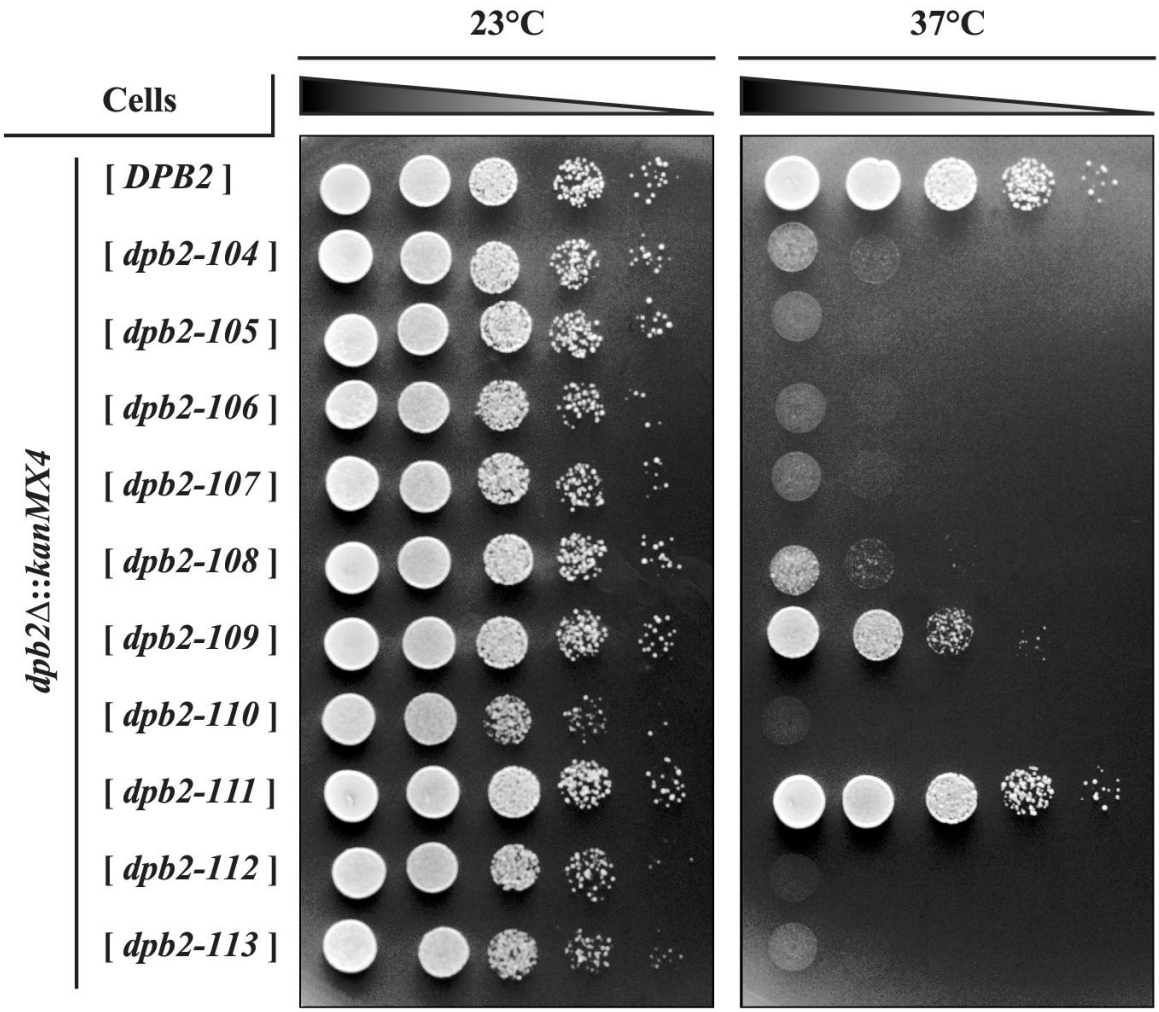


Figure 2. Temperature sensitivity of the *dpb2* mutants

The *dpb2*Δ yeast strains (BY genetic background) bearing individual *dpb2* alleles on a centromeric plasmid were grown at 23°C for 3 days in the SD minimal medium supplemented with required amino acids and nucleotides. Cultures were diluted to identical initial concentrations, 10-fold serial dilutions were spotted in 10-μl portions on SD plates, and the ability to grow at 37°C was subsequently tested. Plates were incubated for 60 h. A strain carrying the wild-type *DPB2* allele on a plasmid was used as a control.

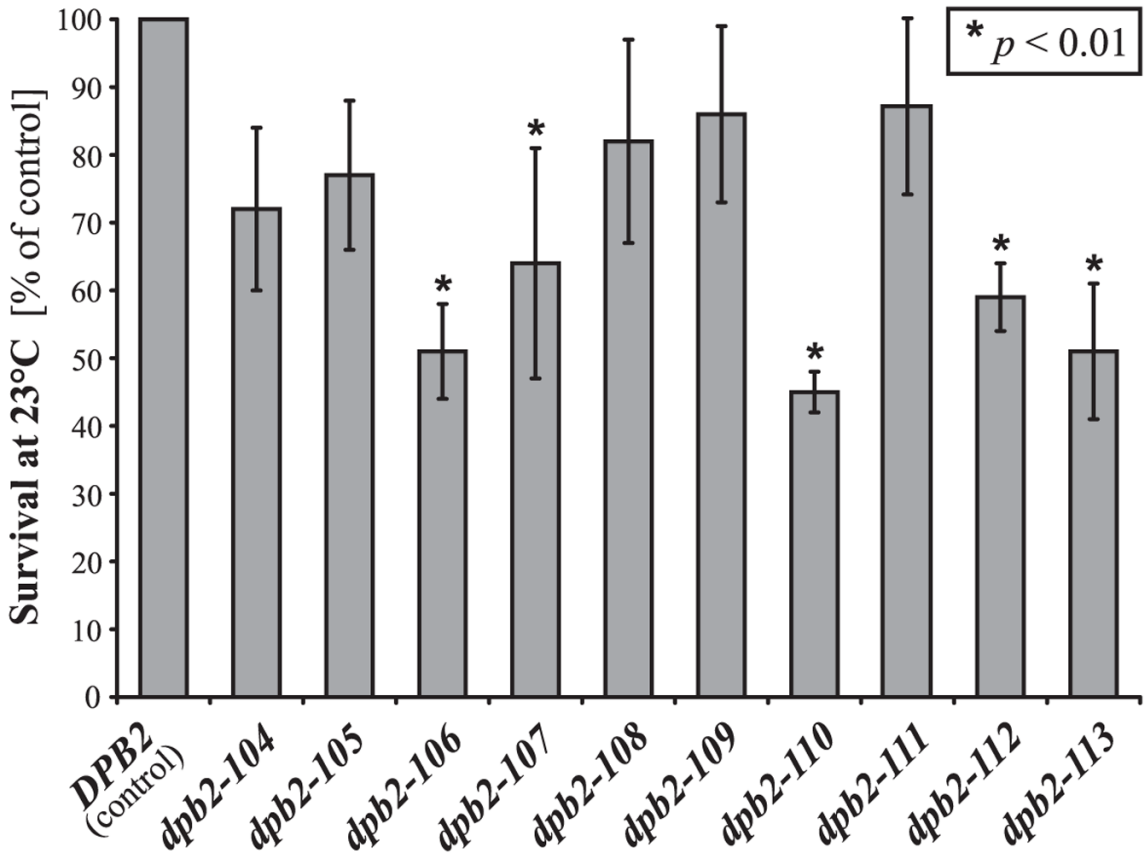


Figure 3. Survival of the temperature-sensitive *dpb2* strains at 23°C

Cultures of the *dpb2*Δ strains carrying the indicated allelesof *DPB2* on centromeric plasmids were grown to stationary phase, yeast cells were collected by centrifugation, washed, and resuspended in water to an OD_{600nm} of 2.0. The cell density determined by direct microscopic cell counts for particular mutant cultures were following: 3.1×10^7 (*DPB2*), 2.5×10^7 (*dpb2-104*), 2.9×10^7 (*dpb2-105*), 2.5×10^7 (*dpb2-106*), 2.6×10^7 (*dpb2-107*), 2.7×10^7 (*dpb2-108*), 2.4×10^7 (*dpb2-109*), 2.4×10^7 (*dpb2-110*), 2.9×10^7 (*dpb2-111*), 2.4×10^7 (*dpb2-112*), 2.4×10^7 (*dpb2-113*). Samples of appropriate dilutions were plated to determine the colony-forming units on SD plates supplemented with required amino acids and nucleotides, and incubated for 7 days at 23°C. Colonies were counted and survival of individual *dpb2* strains was calculated as a percentage of the titer of the strain carrying the wild-type allele of *DPB2*. The values are averages for three experiments. An asterisk indicates that the difference in survival between the mutated *dpb2* strain and the control wild-type *DPB2* strain is “statistically significant” at the 1 % level of significance ($p < 0.01$).

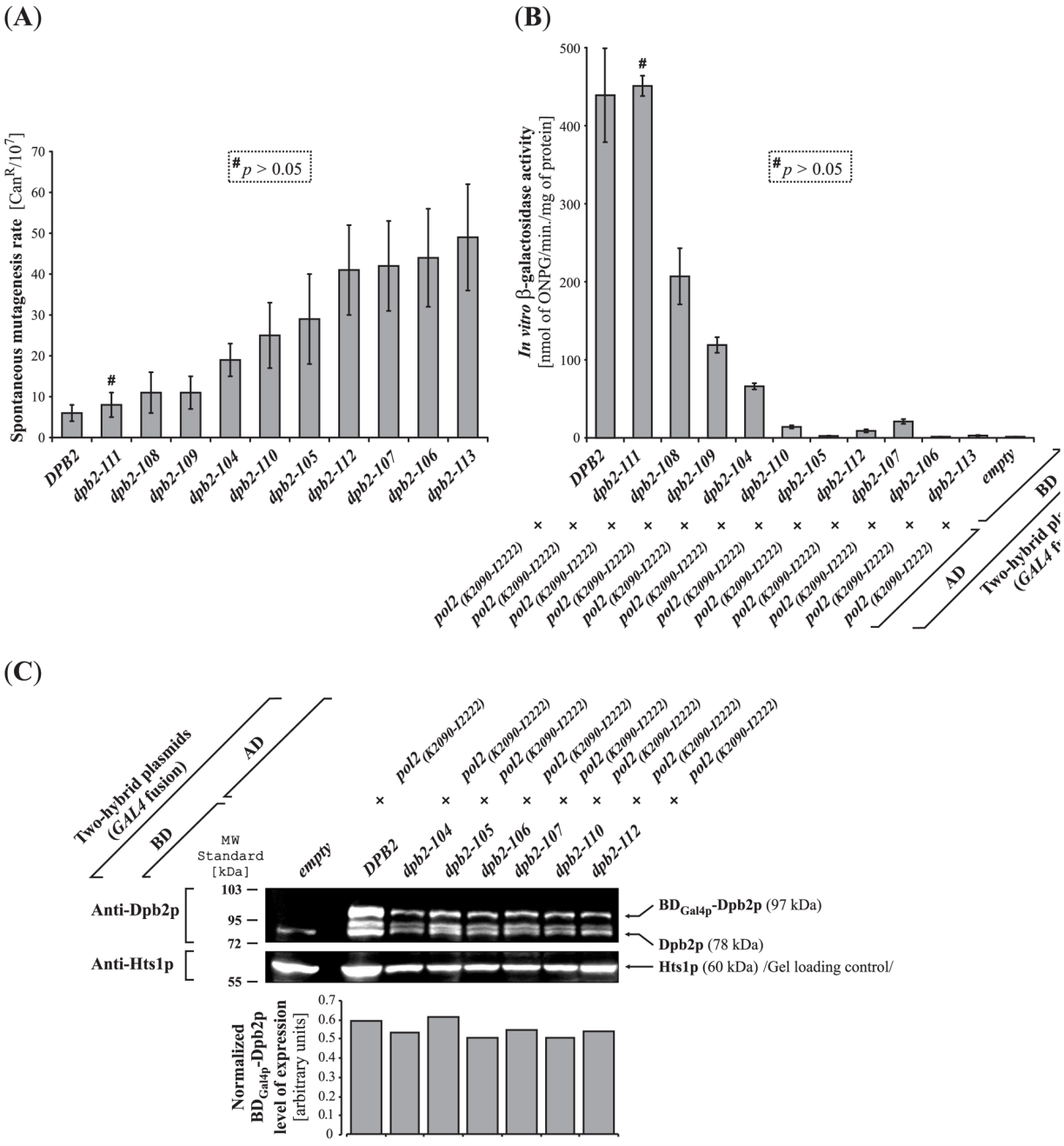


Figure 4. Functional analysis of the *dpb2* alleles: negative correlation between the level of spontaneous mutagenesis and Dpb2p-Pol2p interaction

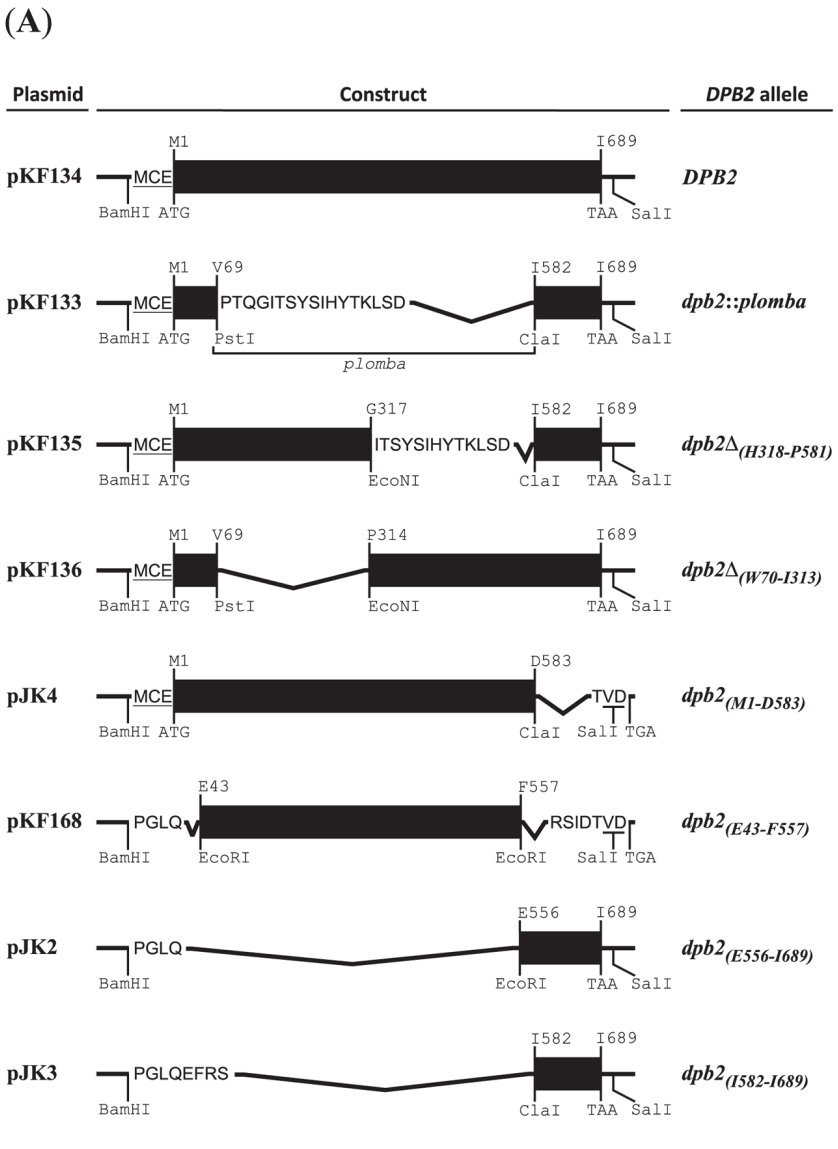
Error bars in histograms A-B indicate standard deviations from three experiments. The values of the spontaneous mutagenesis rate (A) and the *in vitro* β-galactosidase activity (B) in cells bearing the respective *DPB2* variants were compared with corresponding values of cells containing the wild-type *DPB2* (control) at the level of 5 % significance. In the cases where the calculated *p*-value exceeded the set level (indicating that the difference is not "statistically significant" relative to control), the hash (#) sign is presented above the error bar.

A. The *dpb2*-dependent mutator effect. The frequency of forward mutations was measured at the *CAN1* locus as it was described in Materials and Methods. Ten cultures of each strain

were grown to stationary phase, the cells were collected, washed and resuspended in water. Aliquots of undiluted cultures and appropriate dilutions were plated on selective (supplemented with 60 mg/L L-canavanine) and non-selective plates, respectively, and incubated for 7–10 days at 23°C. Colonies were then counted and mutation rates were calculated.

B. Interaction between different mutated variants of Dpb2p and C-terminal part of Pol2p. The Y190 strain was transformed with a pair of the two-hybrid plasmids, as indicated. The interaction between particular Dpb2p variants and the C terminus of Pol2p was tested using *lacZ* genetic reporter encoding β-galactosidase. The yeast strains were grown at 23°C in liquid SD medium supplemented with required amino acids and nucleotides, and the β-galactosidase activity was quantitatively determined using *in vitro* assay and ONPG as a substrate, as described in Materials and Methods. The values are averages for three experiments.

C. Expression of BD_{Gal4p}-Dpb2p fusion variants. Strain carrying wild-type *DPB2* and six strains carrying *dpb2* mutant alleles were used to prepare total protein extracts, and Western blot analysis was performed as described in Materials and Methods. The Dpb2p variants are BD_{Gal4p} fusions and $Pol2p_{(K2090\text{-}I2222)}$ is an AD_{Gal4p} fusion. *Upper panel*: Blot probed with anti-Dpb2p antibodies. The 97-kDa band indicates the BD_{Gal4p}-Dpb2p fusion, whereas the 78-kDa band corresponds to the native Dpb2 protein expressed from a chromosome. *Middle panel*: The same blot probed with anti-Hts1p (histidyl-tRNA synthetase; 60 kDa) [43] antibodies as a gel loading control. *Lower panel*: Comparison of the amount of the respective BD_{Gal4}-Dpb2 fusion proteins, in samples analyzed in the *upper panel*, estimated by quantification of band intensities with the ImageQuant 5.2 densitometry analysis software. The normalized data of the BD_{Gal4p}-Dpb2p expression was calculated by dividing a quantified amount of the respective fusion variant of Dpb2p by the corresponding quantified amount of the Hts1 protein, a gel loading control.



(B)

Interaction of different fragments of Dpb2p with Pol2p

Two-hybrid plasmids (*GAL4* fusion)		*In vitro* β-galactosidase assay*	
BD	AD		
DPB2 **	empty	0.3	(±0.1)
dpb2-100 **	*pol2(K2090-I2222)*	0.3	(±0.1)
empty **		0.5	(±0.1)
DPB2 ***		656.0	(±145.0)
dpb2-102 ***		582.0	(±79.0)
dpb2::plomba		0.3	(±0.1)
dpb2Δ(H318-P581)		0.4	(±0.1)
dpb2Δ(W70-I313)		0.4	(±0.1)
dpb2(M1-D583)		0.4	(±0.1)
dpb2(E43-F557)		0.4	(±0.1)
dpb2(E556-I689)		0.5	(±0.3)
dpb2(I582-I689)		0.4	(±0.1)

* β-galactosidase specific activity was calculated as nmol of ONPG hydrolyzed per min. per mg of proteins; the values are averages for 3 transformants; (±) - standard deviations.

** negative control

*** positive control

Figure 5. Interaction of truncated variants of Dpb2p with a C-terminal part of Pol2p

A. Schematic representation of the truncated *DPB2* alleles cloned into the two-hybrid plasmids. The construction of plasmids with the truncated *DPB2* alleles is described in details in Materials and Methods. All *DPB2* variants were cloned in-frame to the 3′ terminus of the BD_{GAL4} sequence. Fragments of the *DPB2* gene are shown on the scheme as filled boxes. The N- and C-terminal amino acids encoded by the respective *DPB2* fragments are shown above the boxes, while useful restriction sites, as well as START and STOP codons, are shown below the boxes. Additional amino acids encoded by the sequences introduced during cloning processes are indicated using the one-letter code. Although the Dpb2p a.a. numbering system was used according to SGD (i.e. the second in-frame ATG codon was chosen as the translation initiation codon), the full-length *DPB2* open reading frame (ORF) was cloned into pKF75 (yielding pKF134), since the proper initiation codon has not yet been determined experimentally. The possible additional N-terminal a.a. of Dpb2p are underlined.

B. Two-hybrid interactions. The Y190 strain was transformed with the two-hybrid plasmids bearing the truncated variants of the *DPB2* ORF as a fusion with the BD_{GAL4} sequence (pKF133

derivatives), and the AD_{GAL4}-$pol2_{(K2090-I2222)}$ fusion (pKF164). The cultures were grown at 30°C for 24 h and the ability of particular Dpb2p variants to interact with the C terminus of Pol2p was tested using the *lacZ* genetic reporter. The *in vitro* β-galactosidase activity assay was performed as described in Material and Methods. Strains carrying empty vectors or with the mutated *dpb2-100* allele were used as negative controls, whereas strains co-transformed with plasmid pairs bearing (I) the wild-type *DPB2* or mutated *dpb2-102* alleles, and (II) the $pol2_{(K2090-I2222)}$ sequence served as positive controls. The *dpb2-100* and *dpb2-102* alleles, which encode the Dpb2p variants that show respectively, the lack or only slightly reduced interaction with $Pol2p_{(K2090-I2222)}$, were described previously [12].

Table 1

Interaction of different variants of Dpb2p with Pol2p at 23°C and 33°C

Two-hybrid plasmids (*GAL4* fusions)		*In vitro* β-galactosidase activity*	
BD_{GAL4}	**AD_{GAL4}**	**23°C**	**33°C**
DPB2	*empty*	0.5 (±0.1)	1 (±0.1)
empty	$pol2_{(2090\text{-}2222)}$	0.5 (±0.1)	1 (±0.1)
DPB2	$pol2_{(2090\text{-}2222)}$	439 (±119.0)	880 (±26.0)
dpb2-104	$pol2_{(2090\text{-}2222)}$	66 (±4.0)	3 (±7.0)
dpb2-105	$pol2_{(2090\text{-}2222)}$	2 (±0.3)	1 (±0.2)
dpb2-106	$pol2_{(2090\text{-}2222)}$	1.5 (±0.1)	1 (±0.2)
dpb2-107	$pol2_{(2090\text{-}2222)}$	21 (±3.0)	1 (±1.0)
dpb2-108	$pol2_{(2090\text{-}2222)}$	208 (±36.0)	25 (±5.0)
dpb2-109	$pol2_{(2090\text{-}2222)}$	119 (±10.0)	27 (±21.0)
dpb2-110	$pol2_{(2090\text{-}2222)}$	14 (±2.0)	2 (±0.6)
dpb2-111	$pol2_{(2090\text{-}2222)}$	451 (±13.0)	546 (±58.0)
dpb2-112	$pol2_{(2090\text{-}2222)}$	9 (±2.0)	1 (±0.1)
dpb2-113	$pol2_{(2090\text{-}2222)}$	3 (±0.7)	2 (±0.8)

* β-galactosidase activity was calculated as nmol of ONPG hydrolyzed per min. per mg of protein; (±) – standard deviations